SuperArray
Bioscience Corporation

GEArray™ Q and S Series KITS

PATHWAY-SPECIFIC GENE EXPRESSION PROFILING SYSTEMS

USER MANUAL
For chemiluminescent detection

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LIMITED PRODUCT WARRANTY FOR GEARRAY™ PRODUCTS

This product is intended for research purposes only and is not intended for diagnostic purposes or for human use. Most of the cDNA fragments spotted on the GEArray™ membrane products are generated utilizing computer algorithm-designed gene-specific primers and RT-PCR. Their identities are then verified by using a nested PCR technique but not by automated sequencing. The nature of gene array technology in general, including all GEArray™ product lines, necessitates the validation of any array result by an independent method such as Northern blotting or RT-PCR. This warranty limits our liability to replace this product in the event the product fails to perform due to a physical manufacturing defect but not due to an inability to validate by an independent method any results obtained using this product. SuperArray Bioscience Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose. SuperArray Bioscience Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use this product. Warranties are subject to change at any time without notice.

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I. Introduction

The advancement of nucleic acid array technology has made it possible to analyze the expression of multiple genes in a single experiment. However, most commercial gene expression array products, designed to include as many genes as possible, produce vast amounts of data that is overwhelming and difficult to interpret. In addition, the cost of detection equipment and bioinformatics software is often prohibitive.

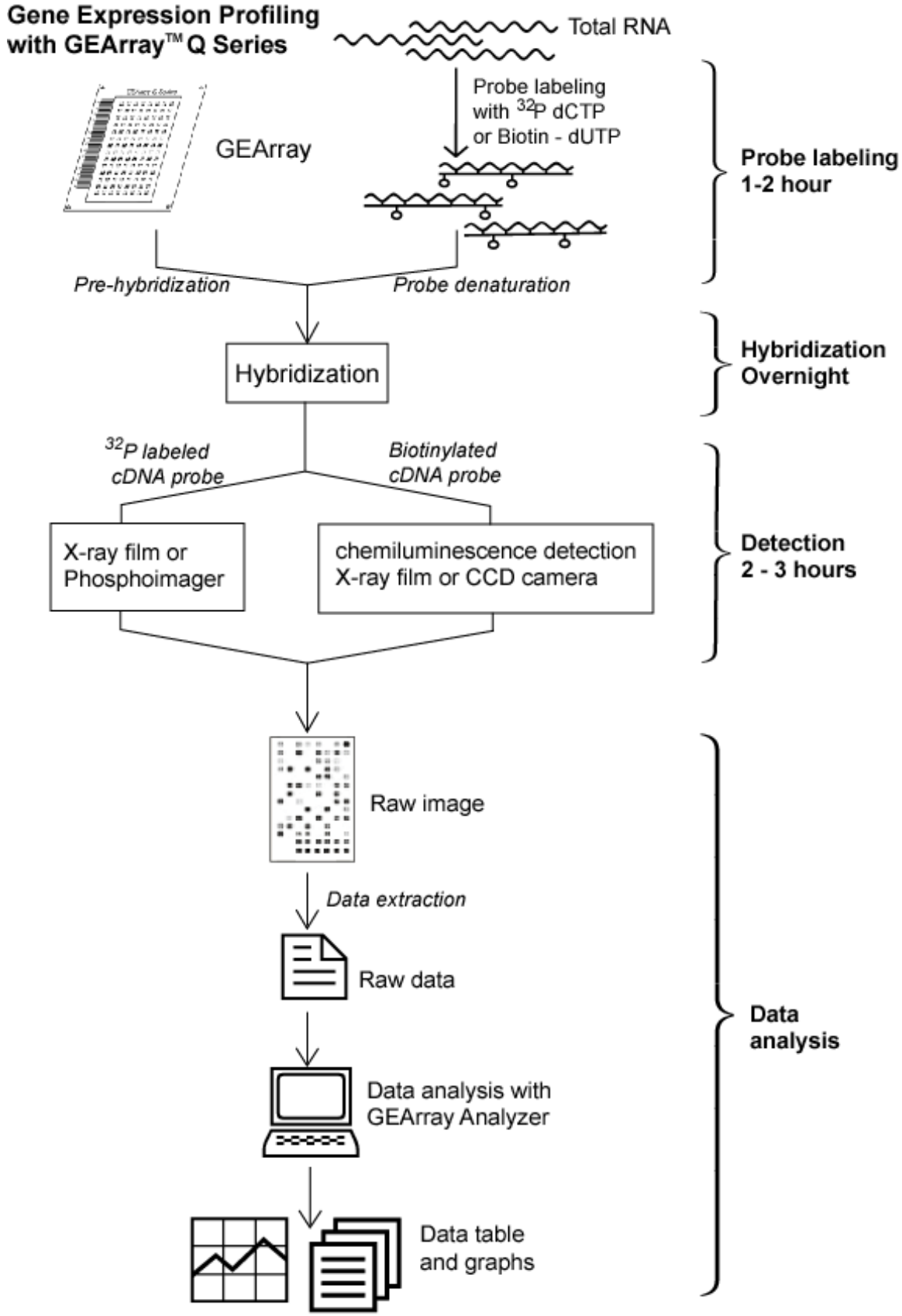
Based on our popular first generation of pathway-specific gene expression arrays, the GEArray™ Q and S Series provide additional features that allow researchers to characterize gene expression associated with a specific biological pathway in a more comprehensive and cost-effective manner. The GEArray™ Q and S Series are a result of combining superior array design with state-of-the-art arraying and detection technologies. They are easy to use, sensitive, economical and accessible for routine use in every research laboratory.

The GEArray™ Q Series gene expression array contains up to 96 and the GEArray™ S Series contains up to 450 cDNA fragments from genes associated with a specific biological pathway. Gene-specific cDNA fragments are printed on a 3.8 x 4.8 cm nylon membrane with an advanced non-contact printing technology. Each cDNA fragment on the Q Series is printed with our tetra-spot format, which provides a signal in an easily identifiable pattern. The cDNA fragments on the S Series are printed in single spots to accommodate an increased number of genes in a grid of 16 columns and up to 30 rows. The catalog number and a unique bar-coded serial number are printed on the reverse side of both the Q and S Series membranes for easy tracking and documentation. The GEArray™ Q and S Series are designed for one-time use only. Each array membrane is packaged in a disposable hybridization tube. Multiple arrays are included in each kit, allowing for a parallel and comparative study of multiple samples. Finally, the GEArray analysis software is available free of charge for GEArray users and can be downloaded from our website at www.superarray.com.

Features of the GEArray™ Q and S Series:

- Easy to use with minimal hands-on time
- Comprehensive collection of genes in a specific pathway
- Proprietary arraying/detection technology offers high sensitivity
- Tetra-spot format for reliable signal detection and identification (***Q Series Only***)
- Available for both radioactive and non-radioactive detection
- No special equipment required
- Cost-effective for routine use
- Free software for data analysis and reporting

Figure 1: Overview of the GEArray™ Q and S Series procedure.
Note: *The spotting pattern on the S series membrane is a 16x18 to 16x30-grid of single spots.*



II. MATERIALS PROVIDED:

GEArray Q or S Series	Trial	Standard	Bulk
GEArray Q or S Series membranes in disposable tubes	2	4	12
BOX 1			
cDNA Probe Synthesis			
Buffer A* (GEAprimer mix) in CLEAR tube	20 µl	20 µl	3x20 µl
Buffer AF* (GEAprimer mix) (**For AmpoLabeling-LPR Kit**) in BLUE tube	40 µl	40 µl	3x40 µl
Hybridization and Detection			
GEAhyb Hybridization Solution	50 ml	50 ml	50 ml
BOX 2			
GEAblocking Solution Q	30 ml	30 ml	30 ml
AP-Streptavidin	20 µl	20 µl	20 µl
5X Buffer F (5X Washing Buffer)	100 ml	100 ml	100 ml
Buffer G (AP Assay Buffer)	100 ml	100 ml	100 ml
CDP-*Star* Substrate	5 ml	5 ml	16 ml

* If using the RT-Labeling Kit (L-01) or the TrueLabeling-RT Kit (L-02) protocols for Probe Synthesis, make sure to use Buffer A. If using the AmpoLabeling-LPR Kit (L-03) protocol for Probe Synthesis, make sure to use Buffer AF.

NOTE: *If using another kit to amplify your RNA, be sure to obtain instructions for generating labeled probe from our website* (www.superarray.com/answer.php?ID=R3).

Please check your Probe Synthesis Kit and its User Manual for other materials mentioned in this GEArray User Manual and required for the protocols within.

Note for Customers outside the USA:

As of September 15, 2003, all other buffers required for probe synthesis are sold separately from you GEArray kit. Please also order either the AmpoLabeling-LPR Kit (Catalog Number L-03) OR the RT-Labeling Buffers (Catalog Number L-04).

III. ADDITIONAL MATERIALS REQUIRED:

A. Software:
The GEArrayAnalyzer software can be downloaded from www.superarray.com free of charge. If you prefer, please call us, and a CD can be sent to you (Cat. No. GA001).

B. Probe Synthesis Kit: Choose **one** of the following options available from SuperArray:

Option 1. Catalog Number L-01 RT-Labeling Kit:
Used for experiments optimized with older GEArray kits.
Available to:
US Customers and Customers Outside the USA **with** a local distributor

Option 2. Catalog Number L-02 TrueLabeling-RT Kit:
Reduces high background and false positive signals.
Available to:
US Customers and Customers Outside the USA **with** a local distributor

Option 3. Catalog Number L-03 AmpoLabeling-LPR Kit:
Reduces high background and false positive signals. Detects lower abundance messages. Provides the most accurate representation of your RNA in gene expression profiles.
Available to:
US Customers and Customers Outside the USA **with** a local distributor
Customers Outside the USA **without** a local distributor in a **modified form**

Alternative Option. Catalog Number L-04 RT-Labeling Buffers:
Used with RNase Inhibitor and MMLV Reverse Transcriptase purchased from other suppliers. Follows same protocol as Option 1, the RT-Labeling Kit.
Available to:
US Customers and Customers Outside the USA **with** a local distributor
Customers Outside the USA **without** a local distributor in a **modified form**

C. Miscellaneous Molecular Biology Reagents and Equipment:
The following reagents are required but are not supplied by SuperArray.

1. cDNA Probe Synthesis:
Biotin-16-dUTP (Roche, Cat. No. 1-093-070).

2. Hybridization and Washing:
Sheared Salmon Sperm DNA (Invitrogen/Life Technologies, Cat. No. 15632-011).
20X SSC: Dissolve 175.3 g NaCl and 88.2 g sodium citrate dihydrate into 900 ml H_2O.
Adjust pH to 7.0 with 1M HCl.
Dilute to 1 L with H_2O. Store at room temperature.
20% SDS: Dissolve 200 g sodium dodecyl sulfate in 1 L H_2O.
Heat to 65 °C if necessary to dissolve. Store at room temperature.

3. You will also need the following laboratory equipment:
Thermal cycler or at least two water baths or hot blocks
Hybridization oven and cylinders or other means to agitate at high temperature
X-ray film (Kodak X-OMAT) and Scanner OR CCD Camera and its software

IV. GEArray™ Q AND S SERIES ASSAY PROTOCOL

Notes: RNA Preparation

Total RNA prepared using commercially available kits is suitable for GEArray™ analysis. Total RNA prepared by Trizol Reagent (Invitrogen/Life Technologies, Cat. No. 15596-018), RNeasy mini kits (Qiagen, Cat. No. 74104) or RNAqueous (Ambion, Cat. No. 1911, 1912, or 1913) gives good results. Total RNA should be dissolved in RNase-Free H_2O. It is not necessary to prepare poly $(A)^+$ RNA; however, the quality of the RNA is crucial for the success of the assay. It is also not necessary to treat RNA samples with DNase. In fact, DNase **should not be used** if you are synthesizing probe with the AmpoLabeling-LPR Kit.

It is important to check the absorbance reading of your RNA sample not only to determine the concentration but also to assess its purity from proteins and free nucleotides. The ratio of the absorbance reading at 260 nm to the reading at 280 nm should be greater than 1.8. In addition and if possible, the integrity of the RNA preparation should be checked by ethidium bromide-stained agarose gel electrophoresis. A quality RNA preparation should yield two sharp bands representing the 28S and the 18S ribosomal RNA (rRNA). The intensity of the 28S (slower mobility) band should also be roughly twice that of the 18S (greater mobility) band. A smearing of either two bands or a decrease in the intensity ratio indicates RNA degradation.

Sometimes even intact RNA does not guarantee good array results because RNA samples may also be contaminated with enzyme inhibitors that can reduce labeling efficiency. We recommend that first-time users of our array kits check their probe labeling before hybridization. (See the Troubleshooting Guide.)

Notes: Description Of Membrane Appearance

Each GEArray Q and S Series nylon membrane array is supplied in a disposable hybridization tube. One side of the membrane is printed with the cDNAs in an 8x14 grid of tetra-spots (Q Series) or a 16x up to 30-grid of single spots (S Series) visible initially as blue dots. These dots will fade by the end of the procedure, and this side of the membrane will then appear blank. This side of the membrane should always face the inside of the hybridization tube, and should face your film or CCD camera during image acquisition. On the reverse side of the membrane, every array has a unique barcode number to allow tracking of the array during the course of your experiment. **Before starting your experiment, always record in your notebook the array bar code number, which contains the array lot number.**

Array side: *Arrayed with cDNA spots. Faces the inside of the hybridization tube.*

Reverse side: *Printed with information. Faces the outside of the hybridization tube.*

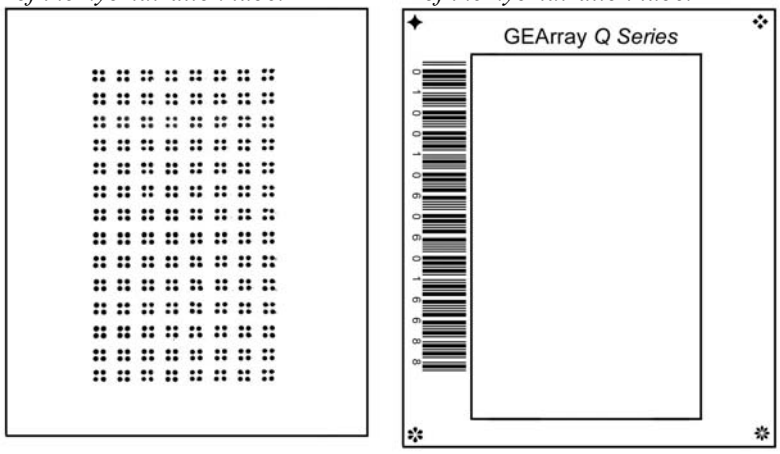


Figure 2: The two sides of the GEArray™ Q Series membrane

Note for S Series users: *The spotting pattern on your membrane is a 16x18- to 16x 30-grid of single spots.*

Note: *Wear gloves while performing all steps of this protocol.*

OPTION #1 (of 3) for Probe Synthesis:

RT-Labeling Kit Protocol (Catalog Number L-01)
Synthesis of cDNA probes with Biotin-16-dUTP (Roche, Cat. No. 1-093-070)

Note: *If you plan to perform your hybridization immediately after probe synthesis, start the pre-hybridization* (**B, step 1, p. 15**) *before continuing with this section.*

International customers: *At this time, the RT-Labeling Kit is only available in international territories with local distributors. Other international customers may follow the notes below.*

Note: *Any customer may also follow this protocol using other sources of RNase Inhibitor (Promega, Catalog Number N2511) and MMLV reverse transcriptase (Promega Catalog Number M1701) together with SuperArray's RT-Labeling Buffers (Catalog Number L-04, sold separately and available to customers inside and outside the USA).*

International customers <u>without</u> a local distributor *will also need to convert 10X* ***RT*** *Buffer from the RT-Labeling Buffers (L-04) into Buffer* ***BN*** *for Chemiluminescent Detection using the following recipe: Combine 50 µL of 10X* ***RT*** *Buffer, 1 µL of 1 M DTT, and 50 µL of dNTP mix (5 mM each dATP, dCTP, and dGTP, and 0.5 mM dTTP). Mix well. Store at –20 °C.*

Note: *There is no need to treat total RNA with DNase.*

a. Prepare the Annealing Mixture:

For each total RNA sample, combine the following in a sterile PCR tube:

Total RNA	1.0-5.0 µg
Buffer **A** (CLEAR tube)	3 µl
RNase-free H_2O	Adjust the final volume to 10 µl.

Note: *Buffer A is unique for each GEArray™ Q and S Series kit. Please do not substitute Buffer A from other GEArray™ Kits. Check the Catalog Number on the tube before use. Also, please do not use any source of Buffer AF in the RT-Labeling Kit Protocol.*

Mix the contents well but gently with a pipettor followed by brief centrifugation. Place the mixture in a pre-heated heat block or thermal cycler at 70 °C for 3 min. Cool to 42 °C and keep at that temperature for 2 min.

Note: *A pre-programmed PCR thermal cycler is recommended.*

b. Prepare the RT Cocktail:

This mixture can be prepared while the Annealing Mixture is incubating at 70 °C.

RT Cocktail

	1 array	2 arrays	4 arrays
Buffer BN	4 µl	8 µl	16 µl
Biotin-16-dUTP	2 µl	4 µl	8 µl
RNase-free H_2O	2 µl	4 µl	8 µl
RNase Inhibitor (RI)	1 µl	2 µl	4 µl
Reverse Transcriptase (RE)	1 µl	2 µl	4 µl
Final volume	10 µl	20 µl	40 µl

Warm the RT Cocktail at 42 °C for 1 min before proceeding to the next step.

c. RT Reaction:

For each array, transfer 10 µl of the pre-warmed RT Cocktail to the 10 µl Annealing Mixture. Mix well but gently with a pipettor. Continue incubation at 42 °C for 90 min. Stop the RT Reaction by adding 2 µl of Buffer C.

Note: *We highly recommend checking the probe for dUTP incorporation at this point before denaturing the probe and setting up the hybridization. See the Troubleshooting Guide at the end of the Manual* (**V. 3.**) *for details.*

d. Denature the probe:

Add 2 µl of Buffer D to each RT Reaction now containing labeled cDNA probe. Incubate at 68 °C for 20 min. Add 25 µl of Buffer E and continue incubation at 68 °C for another 10 min.

Note: *Buffer E may precipitate when thawed at room temperature. We recommend warming Buffer E to 68 °C and vortexing before use.*

Alternatively, the cDNA probe can be denatured by heating at 94 °C for 5 min, and quickly chilling on ice.

Note: *It is not necessary to remove unincorporated biotin-16-dUTP from the cDNA probe. The total labeling reaction mixture can be used for hybridization directly.*

The RT-Labeling probe synthesis protocol is now complete. The cDNA probe is now ready for hybridization (**B**). **Please proceed to page 15.**

OPTION #2 (of 3) for Probe Synthesis:

TrueLabeling-RT Kit Protocol (Catalog Number L-02)
Synthesis of cDNA probes with Biotin-16-dUTP (Roche, Cat. No. 1-093-070)

Note: *If you plan to perform your hybridization immediately after probe synthesis, start the pre-hybridization* (**B, step 1, p. 15**) *before continuing with this section.*

International customers: *At this time, the TrueLabeling-RT Kit is only available in international territories with local distributors. Other international customers should use Option 3 or the Alternation Option. (See the* **Additional Materials Required** *Section.)*

Note: *There is no need to treat total RNA with DNase.*

a. Prepare the Annealing Mixture:
For each total RNA sample, combine the following in a sterile PCR tube:

Total RNA	2.5–5.0 µg
Buffer **A** (CLEAR tube)	3 µl
RNase-free H_2O	Adjust the final volume to 10 µl.

Note: *Buffer A is unique for each GEArray™ Q and S Series kit. Please do not substitute Buffer A from other GEArray™ Kits. Check the Catalog Number on the tube before use. Also, please do not use any source of Buffer AF for the TrueLabeling-RT Kit Protocol.*

Mix the contents gently with a pipettor followed by brief centrifugation. Place the mixture in a pre-heated heat block or thermal cycler at 70 °C for 3 min. Cool to 42 °C and keep at that temperature for 2 min.

Note: *A pre-programmed PCR thermal cycler is recommended.*

b. Prepare the RT Cocktail:

This mixture can be prepared while the Annealing Mixture is incubating at 70 °C.

RT Cocktail

	1 array	2 arrays	4 arrays
Buffer BN	4 µl	8 µl	16 µl
Biotin-16-dUTP	2 µl	4 µl	8 µl
RNase-free H_2O	2 µl	4 µl	8 µl
RNase Inhibitor (RI)	1 µl	2 µl	4 µl
Reverse Transcriptase (AE)	1 µl	2 µl	4 µl
Final Volume	10 µl	20 µl	40 µl

Warm the RT Cocktail at 42 °C for 1 min before proceeding to the next step.

c. RT Reaction:

For each array, transfer 10 µl of the pre-warmed RT Cocktail to the 10 µl Annealing Mixture. Mix well but gently with a pipettor and incubate at 42 °C for 90 min. Stop the RT Reaction by adding 2 µl of Buffer C.

Note: *We highly recommend checking the probe for dUTP incorporation at this point before denaturing the probe and setting up the hybridization. See the Troubleshooting Guide at the end of the Manual* (**V. 3.**) *for details.*

d. Denature the probe:

Add 2 µl of Buffer D to each RT Reaction now containing labeled cDNA probe. Incubate at 68 °C for 20 min. Add 25 µl of Buffer E and continue incubation at 68 °C for another 10 min.

Note: *Buffer E may precipitate when thawed at room temperature. We recommend warming Buffer E to 68 °C and vortexing before use.*

Alternatively, the cDNA probe can be denatured by heating at 94 °C for 5 min, and quickly chilling on ice.

Note: *It is not necessary to remove unincorporated biotin-16-dUTP from the cDNA probe. The total labeling reaction mixture can be used for hybridization directly.*

The TrueLabeling-RT probe synthesis protocol is now complete. The cDNA probe is now ready for hybridization (**B**). **Please proceed to page 15.**

OPTION #3 (of 3) for Probe Synthesis:

AmpoLabeling-LPR Kit Protocol (Catalog Number L-03)
Synthesis of cDNA probes with Biotin-16-dUTP (Roche, Cat. No. 1-093-070)

Note: *If you plan to perform your hybridization immediately after probe synthesis, start the pre-hybridization* (**B, step 1, p. 15**) *before continuing with this section.*

Note: *In order for LPR to work properly, your RNA samples must be free of fragmented genomic DNA. We do not recommend treating your sample with DNase because the fragmented genomic DNA may increase the background. Instead, take care to remove genomic DNA intact from your sample during your isolation protocol.*

Customers outside the USA without a local distributor: *The **RE** and **RI** components below are not included with the kit. Please order MMLV reverse transcriptase and RNasin® Ribonuclease Inhibitor (Promega Cat Nos. M1701 and N2511) and use in the place of **RE** and **RI,** respectively. You also need to convert 10X **RT** Buffer into Buffer **BN**: Combine 50 µL of 10X **RT** Buffer, 1 µL 1 M DTT, and 50 µL dNTP mix (5 mM each dATP, dCTP, and dGTP, and 0.5 mM dTTP). Mix well. Store at –20 °C.*

RT Reaction:

a. Prepare the Annealing Mixture:
For each total RNA sample, combine the following into a sterile PCR tube:

Total RNA*	0.5-5.0 µg
Buffer P	1 µl
RNase-free H_2O	Adjust the final volume to 10 µl.

*We have successfully synthesized labeled probe with as little as 0.1 µg total RNA.

Mix the contents gently with a pipettor followed by brief centrifugation. Place the mixture in a thermal cycler at 70 °C for 3 min. Cool to 37 °C and keep at that temperature for 10 min.

b. Prepare the RT Cocktail:
This mixture can be prepared while the Annealing Mixture is incubating at 37 °C.

RT Cocktail

	1 array	2 arrays	4 arrays
Buffer BN	4 µl	8 µl	16 µl
RNase-free H_2O	4 µl	8 µl	16 µl
RNase Inhibitor (RI)	1 µl	2 µl	4 µl
Reverse Transcriptase (RE)	1 µl	2 µl	4 µl
Final Volume	10 µl	20 µl	40 µl

Warm the RT Cocktail at 37 °C for 1 min before proceeding to the next step.

c. RT Reaction
For each array, transfer 10 µl of the RT Cocktail to the 10 µl Annealing Mixture. Mix well but gently with a pipettor and continue incubation at 37 °C for 25 min. Heat at 85 °C for 5 min to hydrolyze the RNA and to inactivate the reverse transcriptase. Hold the finished RT Reaction on ice until the next step.

LPR Labeling Reaction:

Note: *The temperature should not exceed 85 °C at anytime during LPR.*

d. Prepare the LPR Cocktail:

For each ***Q or S Series kit***, mix the following in a sterile PCR tube:

LPR Cocktail

	1 array	2 arrays	4 arrays
Buffer L	18 µl	36 µl	72 µl
Buffer **AF** (BLUE tube)	9 µl	18 µl	36 µl
Biotin-16-dUTP	2 µl	4 µl	8 µl
DNA Polymerase (LE)	1 µl	2 µl	4 µl
Final Volume	30 µl	60 µl	120 µl

Note: *Buffer AF is unique for each GEArray™ Q and S Series kit. Please do not substitute Buffer AF meant for other GEArray™ Kits. Check the Catalog Number on the tube before use. Also, please do not use any source of Buffer A for the AmpoLabeling-LPR Kit Protocol.*
NOTE: *If using another kit to amplify your RNA, be sure to obtain instructions for generating labeled probe from our website* (www.superarray.com/answer.php?ID=R3).

e. Linear Polymerase Reaction (**LPR**):

For each array, add 30 µl of the LPR Cocktail to each RT Reaction and mix well but gently with a pipettor. Program the thermal cycler for LPR as follows:

85 °C, 5 min; 30 cycles** of (85 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min); then 72 °C, 5 min
**The cycle number can be reduced when using larger amounts of total RNA and/or when specifically interested in more abundant messages. We do not recommend increasing the number of cycles.

f. Immediately stop LPR by adding 5 µl of Buffer C and chilling on ice.

Note: *We highly recommend checking the probe for dUTP incorporation at this point before denaturing the probe and setting up the hybridization. See the Troubleshooting Guide at the end of the Manual* (**V. 3.**) *for details.*

g. Denature the probe:

Denature the labeled cDNA probe by heating the tube containing the LPR at 94 °C for 2 min, and quickly chilling on ice.

Note: *It is not necessary to remove unincorporated biotin-16-dUTP from the cDNA probe. The total labeling reaction mixture can be used for hybridization directly.*

The AmpoLabeling-LPR probe synthesis protocol is now complete. The cDNA probe is now ready for hybridization (**B**). **Please proceed to page 15.**

B. Hybridization:

Note: *To prevent leakage, always tighten the caps of both your hybridization cylinders (that come with your hybridization oven) and our hybridization tubes containing the arrays.*
Note: *An O-ring is provided with the hybridization tube and should be placed around the end of the tube opposite the cap. The O-ring allows the tube to sit level inside your hybridization cylinder and insures that all volumes of solutions cover the membrane evenly.*

1. **Pre-hybridization:**
   Note: *This step can be performed during* **Probe Synthesis** (**A**, p. 9, 11, **OR** 13).

Before starting your experiment, always record in your notebook the array bar code number, which contains the array lot number.

Pre-wet the array membrane by adding roughly 5 ml of deionized water to the hybridization tube. Allow tube to sit inverted while preparing the GEAprehyb.

Warm the GEAhyb Hybridization Solution to 60 °C and invert the bottle several times to allow complete dissolution of the buffer components.

Heat the sheared salmon sperm DNA at 100 °C for 5 min and immediately chill on ice.

Prepare GEAprehyb: Add the heat-denatured salmon sperm DNA to the pre-warmed GEAhyb Hybridization Solution to a final concentration of 100 μg/ml. You will need 3 ml of GEAprehyb for each array. Keep the GEAprehyb solution at 60 °C until needed.

Discard the deionized water from the hybridization tube. Add 2 ml of the GEAprehyb solution, and vortex the tube gently for a few seconds. Be sure the cap of the tube is screwed on hand-tight.

Place the tube inside your hybridization cylinder. Two GEArray Q or S Series hybridization tubes will fit inside a standard hybridization cylinder (ID x L = 35 x 150 mm).

Pre-hybridize in your hybridization oven at 60 °C for 1 to 2 hours with continuous agitation at 5 to 10 rpm.

2. **Hybridization:**

Prepare GEAhyb: Add the entire volume of denatured cDNA probe to 0.75 ml of pre-warmed GEAprehyb. Mix well, and keep the GEAhyb at 60 °C.

Discard the GEAprehyb from the hybridization tube.

Add the GEAhyb (containing probe) to the hybridization tube.

Hybridize overnight at 60 °C with continuous agitation at 5 to 10 rpm.

3. Washing:

Prepare excess: Wash Solution 1: 2X SSC, 1 % SDS
(100 ml 20X SSC and 50 ml 20 % SDS per liter)

Wash Solution 2: 0.1X SSC, 0.5 % SDS
(5 ml 20X SSC and 25 ml 20% SDS per liter)

You will need at least 10 ml of each per hybridization tube. Warm both to 60 °C.

Pour the GEAhyb solution from the hybridization tube into a clean micro-centrifuge tube. Store at 4 °C until the end of the experiment in case another array needs to be probed.

Wash the membrane twice with 5 ml Wash Solution 1 at 60 °C with 20 to 30 rpm agitation for 15 minutes each. Vortex the tube gently with each wash.

Wash the membrane twice with 5 ml Wash Solution 2 at 60 °C with 20 to 30 rpm agitation for 15 minutes each. Vortex the tube gently with each wash.

C. Chemiluminescent Detection

Note: *All of the following detection steps are performed at room temperature.*

Note: *GEAblocking Solution Q and 5X Buffer F may cloud during storage at 4 °C. Warm the solutions to 37 °C and invert the bottles several times to allow any precipitate to completely dissolve. Allow the solutions to sit at room temperature until needed.*

1. **Blocking the Array:**

   After discarding the last wash, immediately add 2 ml GEAblocking Solution Q. Incubate for 40 min with continuous agitation at 20 to 30 rpm.

2. **Binding of alkaline phosphatase-conjugated streptavidin (AP):**

   Prepare Binding Buffer:

Dilute 5X Buffer F five-fold to prepare excess 1X Buffer F. Dilute AP 1:7,500 into 1X Buffer F to obtain your binding buffer. We suggest dispensing volumes of AP no smaller than 2 µl. You will also need 16 ml of 1X Buffer F per tube for washing (**3**).

   Discard the GEAblocking Solution Q from the tube. Add 2 ml Binding Buffer, and incubate for 10 min with continuous but gentle (5-10 rpm) agitation.

3. **Washing:**

   Wash the membrane four times with 4 ml 1X Buffer F for 5 min with gentle agitation. Vortex the tube gently after each addition of fresh 1X Buffer F. Rinse or wash twice with 3 ml Buffer G.

4. **Detection:**

   Add 1.0 ml CDP-Star chemiluminescent substrate to the hybridization tube. Incubate at room temperature for 2 to 5 min.

   Alternatively, place the GEArray Q or S Series membrane on a sheet of plastic wrap and drop the 1.0 ml of CDP-Star solution onto the membrane.

Note: *It is very important to have the membrane covered evenly with the substrate.*

   Blot the membrane on a piece of filter paper to remove excess CDP-Star Solution.
   Do not let the membrane completely dry out.
   The membrane should be saturated and translucent without any solution dripping from it. Place the membrane into a plastic sheet protector or into a small plastic zip-lock bag and smooth out any bubbles.

D. Image and Data Acquisition and Analysis:

1. Image Acquisition

Note: *Remember the blank side of the array should face your film or camera. Orient the array so that the bar code on the reverse side of the membrane is on the right and the name of the array on the reverse side is on the top as you face it. (See Figure 3.)*

Expose the membrane to X-ray film OR use a CCD camera and imaging station to acquire the image. We recommend obtaining multiple exposures for various times. Start with an initial exposure of 1-2 min on film and 5-10 min using a digital imaging system. The visualization of high abundance messages will require shorter exposures; lower abundance messages, longer exposures. If your CCD camera imaging software allows you visualize when the signals are saturated, obtain the longest exposure without saturating any of the signals. The CDP-Star® substrate reaches peak light emission between 2-4 hr, and persists for several days. Therefore, there is ample time to collect the image, but it should be performed as soon as possible.

If X-ray film is used to record the image, use a scanner to convert the image into a grayscale TIFF file. Be sure to save this file. If using a CCD camera to obtain the image, you may also save the image as a grayscale TIFF file. Alternatively, you may use the imaging station's software to save the image and convert it into numerical data.

2. Data Acquisition

If using a grayscale TIFF image, convert the image of tetra-spots into numerical data using the free *ScanAlyze* software developed by Dr. Michael Eisen and featured as a hyperlink on our website (www.superarray.com). The software saves the numerical data as a tabular file recognizable by Microsoft Excel.

If using your imaging station's software to convert the image into numerical data, be sure to collect data from the entire tetra-spot (Q Series) or single spot (S Series) for each cDNA. Collect the data from left to right and top to bottom to insure that the order will match that of our gene lists. Q Series can also refer to Figure 3. Save the raw data as a Microsoft Excel file.

3. Data Analysis

SuperArray has free data analysis software, *GEArray Analyzer*, available on our website (www.superarray.com). *GEArray Analyzer* matches your raw data table with the gene list for the particular GEArray. It also provides you with a list of background subtraction and data normalization options. It generates data tables and other graphical reports, including bar charts, scatter plots and pseudo-color plots. Please refer to the *GEArray Analyzer* User Manual for more detailed instructions.

Each GEArray™ Q Series membrane is spotted with a negative control of pUC18 DNA, blanks, and housekeeping genes, including β-actin, GAPDH, cyclophilin A and ribosomal protein L13a. All raw signal intensities should be corrected for background by subtracting the signal intensity of a negative control or blank. All signal intensities should also be normalized to that of a housekeeping gene. These corrected, normalized signals can then be used to estimate the relative abundance of particular transcripts.

Note: *At the current time, there is no pUC18 DNA control on the S Series membranes. Use a blank for your background subtraction. The same housekeeping genes ARE included on both Q and S Series membranes. Otherwise, perform the array analysis as described.*

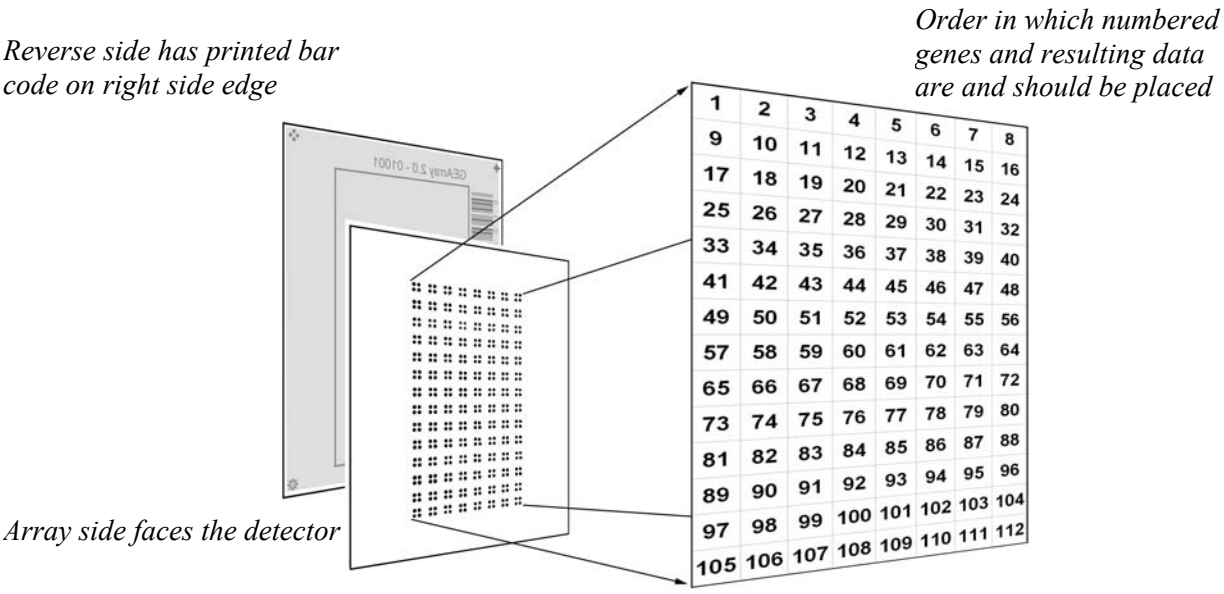


Figure 3: Data extraction sequence for GEArray Q Series
Note for S Series users: *Collect data from the grid of single spots from left to right and top to bottom.*

V. TROUBLESHOOTING GUIDE AND FREQUENTLY ASKED QUESTIONS

RNA quality is crucial to the success of the array. It is important to check your RNA quality. The ratio of the 260 nm and 280 nm absorbance readings should be greater than 1.8. Also, a good RNA prep should produce two sharp bands on an ethidium bromide-stained agarose gel, representing the 28S and the 18S ribosomal RNA (rRNA). The 28S band should be roughly 2 times more intense than the 18S band. A smearing of either of the two bands, a decrease in the 28S to 18S intensity ratio, or a ratio of A_{260}/A_{280} less than 1.8 indicates degradation of the RNA sample. In which case, perform your RNA preparation again before proceeding with the array analysis. However, sometimes even intact RNA **does not** guarantee a good result because the RNA sample may still contain inhibitors that can reduce labeling efficiency. We also recommend that you check the probe labeling before starting the hybridization. (See below.)

A. WEAK OR NO SIGNALS

Other than the quality of RNA sample, the following factors may also impair your results.

1. **Low RNA sample concentration.** If the RNA sample is too dilute, it may not perform well in the assay. The RNA concentration should be at least 0.5 mg/ml.
2. **Inhibited reverse transcriptase.**
   a. Total RNA should be dissolved in RNase-free dH_2O or TE Buffer (10mM Tris-HCl, 1mM EDTA). Be sure that the EDTA concentration does not exceed 1 mM; a high EDTA concentration inhibits the reverse transcriptase.
   b. Solutions that contain SDS as well as unautoclaved DEPC water will inhibit the reverse transcriptase.
   c. If CsCl or LiCl were used in the process of total RNA purification, trace amounts of Cs^+ or Li^+ will inhibit the reverse transcriptase.

3. Insufficient probe labeling.

Many factors including poor RNA quality and contaminants can result in inadequate labeling of the probe. We **highly** recommend checking biotinylated probe labeling efficiency in the following manner:

a. Add 1 µl of the completed but non-denatured Probe Synthesis reaction to 19 µl of 1X agarose gel loading buffer to yield a 20-fold dilution.
b. Perform 4-fold serial dilutions by mixing 3 µl aliquots of each successive dilution with 9 µl of 1X agarose gel loading buffer to yield 80-, 320-, 1280-, and 5120-fold dilutions.
c. Separately spot 1 µl of each diluted sample onto a 3.8 cm x 4.8 cm piece of HyBond nylon membrane (*e.g.* Amersham Pharmacia, Cat. No. RPN303B). Allow the membrane to air-dry for at least 10 minutes.
d. Follow the Chemiluminescent Detection protocol in the GEArray Q Series User Manual for Chemiluminescent Detection. You may even put the membrane in a previously used but clean GEArray Hybridization tube.
e. If the total biotin-deoxyuridylate incorporation is detectable at dilutions of 1000 fold or higher (500 fold or higher for TrueLabeling-RT), the probe should yield good array signals.
f. If you require more reagents, order an Original GEArray Box 2 (Catalog Number 9905300). Note: For these purposes, the GEAblocking buffer for Original and Q Series Kits are interchangeable.

4. Improper washing in 0.1x SSC solution. Excessive washing of the membrane in high stringency buffer (0.1x SSC solution) may strip off the hybridized probes. Washing time should not exceed 15 minutes.

5. Loss of labeled probe during column purification. Column purification can result in a low yield of labeled probe. We recommend adding the complete Probe Synthesis reaction directly into the hybridization solution.

6. Improper hybridization temperature. Check the actual temperature inside your hybridization oven with a thermometer. The temperature reading on your hybridization oven could be several degrees off calibration.

B. HIGH BACKGROUND

Other than the quality of RNA sample, the following factors may cause high background:

1. Pre-hybridization step incomplete. Be sure to denature your sheared salmon sperm DNA completely before making your pre-hybridization solution.

2. Inaccurate AP-streptavidin dilution. Since the AP-streptavidin is dissolved in a glycerol containing solution, special caution should be taken when pipeting the small volume of AP-streptavidin. We suggest diluting no less than 2 µl of AP-streptavidin into 15 ml of buffer (a 1:7,500 dilution). The final working AP-streptavidin dilution can also be increased to 1:12,000.

3. Improper incubation time with AP-streptavidin. Incubation can be reduced to 10 min or less.

4. Improper washing temperature conditions. Be sure to use enough Wash Solution 2 during the low-stringency washing step. Also, be sure to wash the GEArray™ Q or S Series membrane at 60ºC with agitation at 20-30 rpm.

5. **Biotin contamination in containers or buffers.** Milk contains a high amount of biotin. Never use any lab ware that has been used for Western blotting.
6. **Improper membrane incubation.** Always use enough solution to completely cover each membrane. Never let the membranes dry out at any stage in the procedure.
7. **Over-exposure.** Try multiple exposures for various times.
8. **Too much total RNA.** Reduce the amount of total RNA used for Probe Synthesis.

C. How do I prevent leakage of the hybridization tube that you provide?

Put the tube in a regular hybridization cylinder (not provided). Close the cap of this cylinder and our hybridization tube cap hand-tight. An airtight cylinder can prevent the hybridization tube from leaking.

We also suggest using DuraSeal, a sealing film from Diversified Biotech (Cat No. DS1-150 *etc.*). It seals the hybridization tubes very well, which is especially important for users of the Radioactive Detection method.

D. Why do I get a hybridization signal on the negative control (pUC18) spot?

A positive signal on the pUC18 negative control occurs most often when the RNA sample used for Probe Synthesis came from a plasmid transfected cell line. Although we use a gene specific primer mix for the labeling reaction, a small amount of plasmid RNA may still be converted to cDNA and labeled by non-specific priming in the RT reaction. Therefore, it is possible to get hybridization signals on pUC18 spots.

E. The MMLV reverse transcriptase from Promega is 200 Units/µl. Do I need to dilute it to 50 Units/µl before use?

No. Using 5 to 10-fold more reverse transcriptase during Probe Synthesis will not impair your results. However, the concentration of the reverse transcriptase should not be **below** 50 Units/µl.

F. Can I use a reverse transcriptase from another vendor?

Yes, the GEArray labeling buffer works well with reverse transcriptases from many other sources. Make sure that the concentration of your reverse transcriptase is 50 Units/µl or higher.

G. How do I prepare buffer BN?

International customers must convert the 10X RT Buffer included in this kit to Buffer BN. If the Buffer BN included with the Probe Synthesis Kits is stored improperly, domestic customers will need to obtain from us either Buffer BN or 10X RT Buffer in order to regenerate Buffer BN. Use the following recipe:

For every 50 µl 10X RT Buffer, add 1 µl of 1M DTT and 50 µl of 10X dNTP mix (5 mM dATP, 5 mM dCTP, 5 mM dGTP, and *500 µM dTTP*). Mix well. Store the buffer BN at –20 °C.

If you have additional questions, please check our website (www.superarray.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our technical support representatives at 1-888-503-3187 or 301-682-9200.

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